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(54) Title: NON-TOXIC MUCOSAL ADJUVANT

(57) Abstract

A non-toxic mucosal adjuvant is provided which may be admixed with further antigens to provide a vaccine administrable to mucosal surfaces in organisms including man. Preferably, the non-toxic mucosal adjuvant is a detoxified mutant of a bacterial ADP-ribosylating toxin, optionally comprising one or more amino acid additions, deletions or substitutions.

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NONTOXIC MUCOSAL ADJUVANT.

FIELD OF THE INVENTION

The present invention relates to an adjuvant useful for the administration of vaccines to organisms. In particular, the adjuvant of the invention allows the delivery of vaccines to mucosal surfaces to raise a secretory and systemic immune response.

BACKGROUND TO THE INVENTION

Current vaccination technology is based almost exclusively on systemic vaccination techniques wherein the vaccine is injected into the subject to be vaccinated. Only certain live/attenuated vaccines, such as the Sabin polio vaccine, may be taken orally.

The advantages of oral immunisation techniques are several fold. For instance, it is self-evident that a vaccine which may be fed to subjects is easier to administer on a large scale in the absence of specialised equipment, especially to subjects which may be difficult to handle or even locate, such as livestock and wild animals. The spread of infection by the re-use of needles in developing countries would thereby be avoided. Furthermore, an oral vaccine may be provided in the form of an edible solid, which is easier to handle under extreme conditions and is more stable than liquid suspensions as currently used.

Moreover, delivery of immunogens to a mucosal membrane, such as by oral or intranasal vaccination, would permit the raising of a secretory immune response.

The secretory immune response, mainly IgA-mediated, appears to be substantially separate from the systemic immune response. Systemic vaccination is ineffective for raising a secretory immune response. This is a considerable disadvantage when considering immunisation against pathogens, which often enter the subject across a mucosal surface such as the gut or lung.

Unfortunately, it is not possible to raise a secretory immune response to the vast majority of antigens simply by exposing mucosal surfaces to such antigens. Furthermore, no adjuvant capable of eliciting a secretory immune response to a given antigen is currently available.

The apparent difficulty is largely due to a phenomenon known as oral tolerance. The linings of the gut and the lungs are naturally tolerant to foreign antigens, which prevents an immune response being raised to ingested or inhaled substances, such as food and airborne particulate matter.

The ADP-ribosylating bacterial toxins, namely diphtheria toxin, pertussis toxin (PT), cholera toxin (CT), the E.coli heat-labile toxin (LT1 and LT2), Pseudomonas endotoxin A, C. botulinum C2 and C3 toxins as well as toxins from C. perfringens, C. spiriforma and C. difficile are potent toxins in man. These toxins are composed of a monomeric, enzymatically active A subunit which is responsible for ADP-ribosylation of GTP-binding proteins, and a non-toxic B subunit which binds receptors on the surface of the target cell and delivers the A subunit across the cell membrane. In the case of CT and LT, the A subunit is known to increase intracellular cAMP levels in target cells, while the B subunit is pentameric and binds to GM1 ganglioside receptors.

In 1975 and 1978 observations were made which demonstrated that CT is able to induce mucosal and systemic immunity against itself when administered intraduodenally (i.e. to a mucosal surface). The membrane-binding function of CT was shown to be essential for mucosal immugenicity, but cholera toxoid, also known as the B subunit of CT (CTB) was inactive in isolation (Pierce and Gowans, J. Exp. Med 1975; 142: 1550; Pierce, J. Exp Med 1978; 148: 195-206).

Subsequently, it was demonstrated that CT induces systemic and mucosal immunity to co-administered antigens, in other words functions as a mucosal adjuvant (Elson, Curr. Top. Microbiol. Immunal, 1989; 146: 29; Elson and Ealding, J. Immunol. 1984; 133: 2892-2897; Elson and Ealding, Ibid.

1984; 132: 2736-2741; Elson et al., J. Immunol. Methods 1984; 67: 101-118; Lycke and Homgren, Immunology 1986; 59: 301-338).

The experiments referred to above were conducted in mice, which are comparatively resistant to the toxic effects of CT. In contrast, wild-type CT is extremely toxic to humans, rendering the use of CT having any substantial residual toxicity as a mucosal adjuvant in humans entirely out of the question.

Two approaches have been taken in the prior art to address the problem of CT toxicity. The first approach has involved the use of CTB as a mucosal adjuvant. CTB is entirely non-toxic.

In one series of experiments, CTB was covalently coupled to horseradish peroxidase (HRP) and administered to mice intraduodenally. This gave rise to a powerful mucosal immune response to HRP (McKenzie and Halsey, J. Immunol 1984; 133: 1818-1824).

This result has subsequently been partially confirmed with other antigens (Liang et al., J. Immunol 1988; 141: 1495-1501; Czerkinsky et al., Infect. Immun. 1989; 57: 1072-1077). The same principle has also been established to be effective when chimeric antigens produced by gene fusion to sequences encoding CTB have been tested (Dertzbaugh and Elson, Infect. Immun. 1993; 61: 384-390; Dertzbaugh and Elson, Ibid. 1993; 61: 48-55; Sanchez et al., Res. Microbiol 1990; 141: 971-979; Holmgren et al., Vaccine 1993; 11: 1179-1184).

However, the production of chimeric or coupled antigens introduces a further step in the preparation of suitable vaccines, which essentially requires that antigens be prepared in a form conjugated with CTB especially for oral use. It would be for simpler and more economical if the adjuvant could be administered in simple admixture with the antigen.

An adjuvant effect for co-administered CTB has been alleged in a number of publications (Tamura et al., J. Immunol 1992; 149: 981-988; Hirabayashi et al., Immunology

1992; 75: 493-498; Gizurarson *et al.*, Vaccine 1991; 9: 825-832; Kikuta *et al.*, Vaccine 1970; 8: 595-599; Hirabayashi *et al.* Ibid. 1990; 8; 243-248; Tamura *et al.*, Ibid. 1989; 7: 314-32-; Tamura *et al.*, Ibid. 1989; 7: 257-262; Tamura *et al.*, Ibid 1988; 6: 409-413; Hirabayashi *et al.*, Immunology 1991; 72: 329-335 Tamura *et al.*, Vaccine 1989; 7: 503-505).

However, a number of aspects of the observations reported above were not entirely convincing. For example, it was noted that the adjuvant effect ascribed to CTB was not H-2 restricted. It is known, however, that immune response to CTB is H-2 restricted (Elson and Ealding, Eur. J. Immunol. 1987; 17: 425-428). Moreover, the alleged adjuvant effect was observed even in individuals already immune to CTB.

Other groups were unable to observe any mucosal adjuvant effect attributable to CTB (Lycke and Holmgren, Immunology 1986; 59: 301-308; Lycke *et al.*, Eur. J. Immunol. 1992; 22: 2277-2281). Experiments with recombinant CTB (Holmgren *et al.*, Vaccine 1993; 11: 1179-1183) confirmed that the alleged effect is largely if not exclusively attributable to low levels of contamination of CTB preparations with CT.

Thus, it is presently accepted that CTB is not useful as a mucosal adjuvant.

A second approach to eliminating the toxicity of CT has been to mutate the CT holotoxin in order to reduce or eliminate its toxicity. The toxicity of CT resides in the A subunit and a number of mutants of CT and its homologue, LT, comprising point mutations in the A subunit are known in the art. See, for example, International Patent Application WO92/19265 (Amgen). It is accepted in the art that CT and LT are generally interchangeable, showing considerable homology.

However, the only mutant so far tested for mucosal adjuvanticity, an LT mutant having a Glu-Lys mutation at position 112, was found to be inactive as a mucosal adjuvant (Lycke *et al*; Eur. J. Immunol. 1992; 22: 2277-2251; Holmgren *et al.*, Vaccine 1993; 11: 1179-1183). The authors

of these publications conclude that there is a link between the ADP ribosylating activity of CT and/or LT and the adjuvant activity. It appears from these publications, therefore, that CTB or a non-toxic mutant of CT or LT would not be active as a mucosal adjuvant.

SUMMARY OF THE INVENTION

There therefore remains a need for an active mucosal adjuvant which may be used to increase the immunogenicity of an antigen when administered to a mucosal surface, such as orally or intranasally.

It has now been discovered that, in complete contradiction with the results and conclusions presented in the prior art, the toxic and adjuvant activities of the ADP-ribosylating toxins are separable. An entirely non-toxic mutant of such a toxin has been shown to be active as a mucosal adjuvant.

The present invention, in a first aspect, provides a pharmaceutical composition comprising a non-toxic mucosal adjuvant in admixture with a second antigen.

It has been demonstrated that an LT mutant which completely lacks toxicity is active as a mucosal adjuvant and protects subjects against subsequent challenge with a lethal dose of the immunogen. Although the Applicants do not wish to be bound by any particular theory, it is postulated that the results of Lycke *et al.* and Holmgren *et al.* quoted above may be contradicted at least in part because they fail to take into account the stability of the mutant being made. *Inter alia* by ensuring that the non-toxic mutant of the invention is stable at the site of delivery, it has been demonstrated that the adjuvant effect of CT and/or LT may be maintained while its toxic effects are eliminated.

Preferably, therefore, the non-toxic mucosal adjuvant is a detoxified mutant of a bacterial ADP-ribosylating toxin, optionally comprising one or more amino acid additions, deletions or substitutions.

Particularly suitable are detoxified mutants of CT or LT. For example, a mutant LT in accordance with the invention may possess an Arg7 to Lys7 substitution at position 7 of the A subunit, the so-called LTK7 mutant.

Alternative mutants are known to those skilled in the art and are preferred molecules for use in the present invention. Examples include PT mutated at position 129, in particular PT having a Glu 129->Gly mutation. Further mutants include PT mutated at one or both of Trp 26 and Arg 9, optionally in combination with the Glu 129 mutation.

The mutant used in the invention may moreover be a mutant wherein the mutation has been effected in a part of the molecule which results in the prevention of proteolytic cleavage of the A subunit of the toxin, such that enzymatic activity is not brought about. Such mutants are described in Grant *et al*. Inf. and Immunity (1994) 62(10) 4270-4278. For example, the mutant may comprise an Arg 192->Gly mutation in LT or a corresponding mutation in another ADP-ribosylating toxin.

The mutant of the invention is preferably in the form of a holotoxin, comprising the mutated A subunit and the B subunit, which may be oligomeric, as in the wild-type holotoxin. The B subunit is preferably not mutated. However, it is envisaged that a mutated A subunit may be used in isolation from the B subunit, either in an essentially pure form or complexed with other agents, which may replace the B subunit and/or its functional contribution.

Methods for the design and production of mutants of CT and/or LT are known in the art. Suitable methods are described in International Patent Application WO93/13202 (Biocine Sclavo), the disclosure of which is incorporated herein by reference, as well as WO92/19265 (Amgen).

The adjuvant of the invention is preferably administered in admixture with a suitable antigen against which it is desired to raise an immune response. If the antigen and the adjuvant are not in admixture, it is preferred that they be administered within a relatively

short time of each other, at the same site of administration. It has been observed that the adjuvant effect provided by wild-type CT is short lived (see Lycke and Homgren, Immunology 1986; 59: 301-308). In an alternative embodiment, the mucosal adjuvant of the invention may be administered, optionally in isolation from other antigens, as a boost following systemic or mucosal administration of a vaccine.

The precise formulation of the vaccine may vary in accordance with the nature of the immunogen. For example, if the antigen is enclosed in slow-releasing microspheres to liposomes, the mucosal adjuvant may be similarly enclosed so that the antigen and the adjuvant may interact simultaneously with the mucosal immune system. Alternatively, separate mucosal administration of the adjuvant of the invention may be used to enhance mucosal response to parentally-administered vaccines.

In a second aspect, the present invention provides the use of a non-toxic mutant of CT or LT as a mucosal adjuvant in the preparation of a composition for mucosal administration.

Preferably, the composition is a vaccine and is useful for the immunisation of a subject against a disease or the treatment of a subject suffering from a disease.

Preferably, the mutant comprises one or more amino acid additions, substitutions or deletions in the amino acid sequence of the A subunit of CT or LT which is or are effective to abolish the toxicity of the toxin.

According to a third aspect of the invention, there is provided a method for the prevention or treatment of a disease in a subject comprising administering to the subject an immunologically effective dose of an antigen adjuvanted with a non-toxic CT or LT mutant by contacting a mucosal surface of the subject with said adjuvanted antigen.

The mucosal surface may be any suitable mucosal surface of the subject. For example, the administration may be carried out by inhalation, by means of a rectal or vaginal suppository, or a pessary, by feeding or other

buccal administration, by means of an aerosol, by intranasal delivery or direct application to mucosal surfaces. Especially preferred are oral and intranasal administration.

The subject may be any organism susceptible to immunisation. Especially indicated are humans and other mammals such as livestock, pets and wildlife.

Diseases against which the subject may be immunised include all diseases capable of being treated or prevented by immunisation, including viral diseases, allergic manifestations, diseases caused by bacterial or other pathogens which enter through or colonise mucosal surfaces, AIDS, autoimmune diseases such as systemic Lupus Erythe - matosus, Alzheimer's disease and cancers. Examples of viral infections which may be treated or prevented using the invention include infection by DNA viruses, such as EBV and VZV, and in particular herpesviridae, for example HSV and HCMV, adenoviridae, papovaviridae, such as HPV, hepadna -viridae, such as HBV, infection by RNA viruses, such as picorvaviridae, especially polivirus and HAV, rhinoviruses and FMDV, togaviridae, flaviviridae, coronaviridae, paramyxo -viridae, such as RSV, orthomyoxoviridae, such as influenza virus, and retroviridae, especially HIV. Vaccination against HCV and HDV is also envisaged.

Examples of bacterial infections suitable for treatment or prophylaxis by the invention include infection with Helicobacter pylori, streptococci, meningococcus A, B, and C, bordetella pertussis and chlamydia and trachomatis.

Vaccine formulation suitable for delivery at mucosal surfaces may be prepared as set out hereinbelow, while further formulations will be apparent to those of skill in the art. Suitable administration regimes are, likewise, set out below while modifications of the exemplified values will be apparent to those of skill in the art.

Moreover, the invention provides a mutant of CT or LT which is a non-toxic mucosal adjuvant and a second antigen for simultaneous separate or sequential administration. Simultaneous administration of the adjuvant and the second antigen when combined in a single vehicle, carrier or

particle, as exemplified below, is particularly preferred.

The second antigen may be any antigen to which it is desired to raise an immune response in the subject. Suitable antigens comprise bacterial, viral and protozoan antigens derived from pathogenic organisms, as well as allergens, allogens and antigens derived from tumours and self-antigens. Typically, the antigen will be a protein, polypeptide or peptide antigen, but alternative antigenic structures, such as nucleic acid antigens, carbohydrate antigens, and whole or attenuated or inactivated organisms such as bacteria, viruses or protozoa are not excluded. The invention further provides a method for the manufacture of an adjuvanted vaccine comprising the steps of:

a) performing site-diected mutagenesis on the A-subunit of a bacterial ADP-ribosylating toxin in order to detoxify the toxin; and

b) bringing the detoxified toxin into association with a second antigen, such that it functions as a mucosal adjuvant.

Specific examples of antigens useful in the present invention include HSV gD, gB and other glycoproteins; HIV gp120 and other proteins; CMV gB or gH; HCV antigens; HDV delta antigen; HAV antigens; EBV and VZV antigens; B. pertussis antigens and H. pylori antigens.

In general, the second antigen may be the immunogenic component of the vaccine intended for injection. Such vaccines, and the immunogenic components thereof, may be subunit vaccines, whole inactivated or attenuated organisms or polynucleotide vaccines.

The vaccines according to the invention may either be prophylactic (to prevent infection) or therapeutic (to treat disease after infection).

These vaccines may either be prophylactic (to prevent infection) or therapeutic (to treat disease after infection).

Such vaccines comprise antigen or antigens, usually in combination with "pharmaceutically acceptable carriers," which include any carrier that does not itself induce the

production of antibodies harmful to the individual receiving the composition.

Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplet emulsions or liposomes), and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. In preferred aspects of the invention, these carriers may function as immunostimulating agents ("adjuvants"). Furthermore, the antigen may be conjugated to a bacterial toxoid, such as a toxoid from diphtheria, tetanus, cholera, H. pylori, etc. pathogens.

Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to: (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (see below) or bacterial cell wall components), such as for example (a) MF59 (PCT Publ. No. WO 90/14837), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE (see below), although not required) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA), (b) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP (see below) either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) RibiTM adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (DetoxTM); (3) saponin adjuvants, such as StimulonTM (Cambridge Bioscience, Worcester, MA) may be used or particles generated therefrom such as ISCOMs

(immunostimulating complexes); (4) Complete Freunds Adjuvant (CFA) and Incomplete Freunds Adjuvant (IFA); (5) cytokines, such as interleukins (e.g., IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, etc.), interferons (e.g., gamma interferon), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc; and (6) other substances that act as immunostimulating agents to enhance the effectiveness of the composition. Alum and MF59 are preferred.

As mentioned above, muramyl peptides include, but are not limited to, N-acetyl-muramyl-L-threonyl-D -isoglutamine (thr-MDP),N-acetyl-normuramyl-l-alanyl -d-isoglutamine (nor-MDP),N-acetylmuramyl-l-alanyl-d-isoglutaminyl-l-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-huydroxyphosphoryloxy)-ethylamine (MTP-PE), etc.

The immunogenic compositions (e.g., the antigen, pharmaceutically acceptable carrier, and adjuvant) typically will contain diluents, such as water, saline, glycerol, ethanol, etc.

Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles.

Typically, the immunogenic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation also may be emulsified or encapsulated in liposomes for enhanced adjuvant effect, as discussed above under pharmaceutically acceptable carriers.

Immunogenic compositions used as vaccines comprise an immunologically effective amount of the antigenic polypeptides, as well as any other of the above-mentioned components, as needed. By "immunologically effective amount", it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated (e.g., nonhuman primate,

primate, etc.), the capacity of the individual's immune system to synthesize antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

The immunogenic compositions are conventionally administered parenterally, e.g., by injection, either subcutaneously or intramuscularly. Additional formulations suitable for other modes of administration include oral and pulmonary formulations, suppositories, and transdermal applications. Dosage treatment may be a single dose schedule or a multiple dose schedule. The vaccine may be administered in conjunction with other immunoregulatory agents.

Examples of suitable immunostimulatory agents include interleukins, such as interleukins 1,2, 4-7 and 12, and interferons, especially γ-interferon.

The invention is described hereinbelow by way of example only, with reference to the following Figures:-

DESCRIPTION OF THE FIGURES

Figure 1a shows the titre of total ovalbumin specific antibody in BALB/c mice immunised i/n or s/c with either ovalbumin alone or ovalbumin together with toxin derivatives;

Figure 1b shows the titre of total toxin-specific antibody in the mice of Figure 1a;

Figure 2 shows a measurement of ovalbumin-specific IgA in nasal and lung lavages of mice injected as in Figure 1; and

Figure 3 shows the presence of tetanus toxoid-specific antibodies in the serum of BALB/c mice immunised i/n or s/c with tetanus toxin fragment C alone or together with toxin derivatives.

DETAILED DESCRIPTION OF THE INVENTION

Site-directed mutagenesis was used to replace the arginine residue at position seven of the A subunit of LT with lysine in order to construct a non-toxic LT mutant that could still assemble as a holotoxin with cell binding activity. The mutant protein, named LTK7, was purified and tested for ADP-ribosyltransferase and toxic activity in several assays. LTK7 was still able to bind GM1 ganglioside receptor but showed a complete loss of enzymatic activity, in agreement with published data (Lobet *et al.*, Infect. Immun. 1991; 59:2870-2879). Further, LTK7 was inactive in the mouse ileal loop assay and *in vitro* on Y1 cells, even when a dose equivalent to 10^7 toxic units of wild-type LT was tested (Table 1).

In vivo and *in vitro* properties of LT and of LT K-7 mutant

TABLE 1

	LT	LT-K7	LT/LTK7
Codon in position 7 of the A subunit	CGT	AAG	-
Aminoacid in position 7 of the A subunit	Arg	Lys	-
ADP-ribosyltransferase activity of the A subunit	0.06µg	>> 20µg	<< 3.10^{-3}*
In vivo in mouse ileal loop	10µg	>> 500 µg/mouse	<< 0.02 **
In vitro toxicity on Y1 cells	10pg/ml	>> 100 µg/ml	<< 10^{-7} **
Binding to eukaryotic	+	+	1

* Data published by Lobet *et al.* and confirmed in this study

** This study

>> Means that LT-K7 was still enzymatically inactive or non toxic when the highest concentration shown in the table was tested.

<< Indicates that the real difference is higher than the number shown which represents the difference tested.

The ability of LTK7 to act as a mucosal adjuvant was assessed in mice. Mice were separated into groups and immunised using ovalbumin as a reporter antigen. Animals were immunised intranasally (i/n) or subcutaneously (s/c) using 10µg of ovalbumin alone or ovalbumin mixed with either 1µg CT, LT or LTK7. Mice were split into four groups of six mice. Four mice from each group were lightly anaesthetised and immunised with either 10µg of ovalbumin or 10µg of ovalbumin with 1µg of toxins, delivered in a total volume of 30µl. The remaining two mice were immunised with the same amount of proteins s/c in a total volume of 100µl. Proteins given subcut were first adsorbed to 2% $Al(OH)_3$.

Animals were immunised on days 1, 22, 36 and 61.

Sample bleeds of 100µl were collected on day 0, 21, 35, 56 and on day 76 animals were culled by cardiac puncture.

Quantitation of antibody was estimated by ELISA. For estimation of ovalbumin-specific antibodies, 96-well EIA plates (costar) were coated overnight with 60 µg/ml of ovalbumin. Measurement of toxin-specific antibodies was performed using a GM1 capture ELISA. Toxin-specific antibodies were measured against the antigen used in the immunisations. No single toxin was used in the measurements of toxin-specific antibody from each group, and as such the titres between these groups can not be compared directly.

Sera from each group were pooled from four and two mice respectively. Samples were prepared in duplicate from a dilution of 1:50. Absorbences were read at 450nm using the kineticalc version 2.13 programme (Biotek instruments). This programme calculates the rate of change of substrate over thirty time points ten seconds apart.

ELISA titres of antibody were measured arbitrarily as the dilution of serum which gave half the maximal absorbence at 450nm. Sera which failed to show absorbence at 450nm 2.5 times greater than that observed with the equivalent pre-immune sera were considered negative. Results shown in Figure 1a and 1b represent the mean titre values from duplicate wells from one experiment. No significant levels of antibodies to ovalbumin above background were detected in the serum of mice immunised i/n with ovalbumin alone although mice immunised s/c efficiently sero-converted. Mice receiving ovalbumin along with either CT or LT i/n contained very high levels of anti-ovalbumin antibodies in their sera. These were equivalent to those observed when mice immunised s/c. Mice that received ovalbumin with LTK7 also showed very high levels of antibodies to ovalbumin.

The levels of anti-toxoid responses in these same groups are shown in Figure 1b. All mice, including those immunised with the mutant toxin, developed high levels of antibodies to these toxin in their sera.

The local secretory antibody levels to ovalbumin were

measured using both lung and nose washings (Fig. 2). In brief animals were culled by cardiac puncture and dissected so that the trachea was exposed. An ultra-thin pipette was then inserted into a small nick in the trachea. Lung washes were collected by repeated flushing and aspiration of 1.5 ml of 0.1% bovine serum albumin (Sigma), in PBS, into the lungs. Nose washes were collected by flushing 1ml of 0.1% BSA in PBS through the nasal cavity.

Ovalbumin-specific IgA antibodies were measured by ELISA using an anti-mouse alpha-chain-specific conjugate antibody (Serotec). Samples were prepared from individual animals and columns in this figure represent the mean rate of change of substrate, using kineticalc, for four and two mice immunised i/n and s/c respectively. The figures are constructed using the raw absorbence data at a dilution of 1:3 with respect to lung washes. These correspond to titres of between 1:2 and 1:6 for nose washes and between 1:70 and 1:120 for lung washes. These titres were calculated using the method described above. Mice immunised s/c or i/n with ovalbumin alone contained no detectable ovalbumin-specific IgA in the washings sampled. All individual mice immunised with ovalbumin in combination with CT, LT or LTK7, showed detectable levels of anti-ovalbumin IgA. Thus both a local and systemic anti-ovalbumin response are detectable in these animals.

In the face of these encouraging experiments with ovalbumin the immunisation was repeated using Fragment C, a 50,000 dalton, non-toxic portion of tetanus toxin which had been expressed in and purified from the yeast *Pichia pastoris*. Mice were immunised either s/c or i/n with Fragment C alone or mixed with individual samples of either LT or LTK7. Mice were separated into four groups of ten mice and four groups of five mice. Ten mice were immunised i/n with a) 10µg of fragment C alone; b) 10µg of fragment C + 1µg of LT; c) 10µg of fragment C + 1µg of LTK7 and d) PBS only, all in a final volume of 30 µl. Five mice were immunised i/n with a) 1µg of LT and b) 1µg of LTK7. The remaining two groups of mice were immunised s/c with either

no protein or 10μg of fragment C in a dose volume of 100μl. These vaccines were prepared as described in Figure 1. Animals were immunised on day 1 and 22. Sample bleeds of 100μl were collected on day 0, 21 and 35. Fragment C-specific antibodies were measured by ELISA using tetanus toxid (10μg/ml) as the coating antigen. Sera from each group were pooled. Samples were prepared in duplicate from a dilution of 1:50. ELISA titres were calculated as described above. Mice immunised s/c with Fragment C efficiently sero-converted producing high levels of anti-Fragment C antibodies (Fig. 3). Mice immunised i/n with Fragment C alone showed no significant sero-conversion. However mice immunised with Fragment C combined with LT or LTK7 showed high levels of anti Fragment C antibodies in their sera (Fig. 3). Since mice that sero-convert to Fragment C can be protected against toxin challenge the groups were challenged with active tetanus toxin. All mice immunised s/c with Fragment C alone were protected whereas all mice immunised i/n were highly susceptible. All mice i/n immunised with Fragment C combined with either LT or LTK7 survived the challenge (Table 2).

TABLE 2

	Serum anti-Fragment C	Deaths
LT	---	10/10
LTK7	---	10/10
LTK7 + Fragment C	++	0/10
Lt + Fragment C	++++	0/10
Fragment C	+/-	10/10

The titre of anti-Fragment C antibodies in the serum of mice was on average about 1/3,000 in mice vaccinated with the K7 mutant + Fragment C and 1/12,000 for LT + Fragment C.

These experiments show that protective immunity against tetanus can be achieved using a non-toxic LT mutant as adjuvant and that mucosal immunisation with this molecule can generate both local secretory and systemic immune response to the toxin and co-administered bystander antigens.

CLAIMS:

1. A pharmaceutical composition comprising a non-toxic mucosal adjuvant in admixture with a second antigen.

2 A composition according to claim 1 wherein the non-toxic mucosal adjuvant is a detoxified mutant of a bacterial ADP-ribosylating toxin.

3. A composition according to claim 2 wherein the non-toxic adjuvant is a detoxified mutant of CT or LT.

4. A composition according to claim 2 or claim 3 wherein the non-toxic mucosal adjuvant comprises one or more amino acid additions, deletions or substitutions in the A subunit of the holotoxin.

5. A composition according to claim 4 wherein the non-toxic mucosal adjuvant is LTK7.

6. Use of a non-toxic mutant as defined in any one of claims 2 to 5 as a mucosal adjuvant in the preparation of a composition for mucosal administration.

7. Use according to claim 6, wherein the composition is a vaccine.

8. Use according to claim 7, wherein the vaccine is for use in prophylactic or therapeutic applications.

9. Use according to any on of claims 6 to 8, wherein the composition further comprises a second antigen.

10. A method for the prevention or treatment of a disease in a subject comprising administering to the subject an immunologically effective dose of an antigen adjuvanted with a non-toxic mutant as defined in any one of claims 2 to 5 by contacting a mucosal surface of the subject with said adjuvanted antigen.

11. A method according to claim 10 comprising administration of a composition according to any one of claims 1 to 5.

12. A method according to claim 11 or claim 12, wherein the adjuvanted antigen is administered orally or intranasally.

13. A non-toxic mucosal adjuvant and a second antigen for simultaneous separate or sequential administration.

14. A non-toxic mucosal adjuvant and a second antigen for simultaneous administration when combined in a single vehicle, carrier or particle.

15. A method for the manufacture of an adjuvanted vaccine comprising the steps of:

a) performing site-directed mutagenesis on the A-subunit of a bacterial ADP-ribosylating toxin in order to detoxify the toxin; and

b) bring the detoxified toxin into association with a second antigen, such that it functions as a mucosal adjuvant.

FIG. 1a

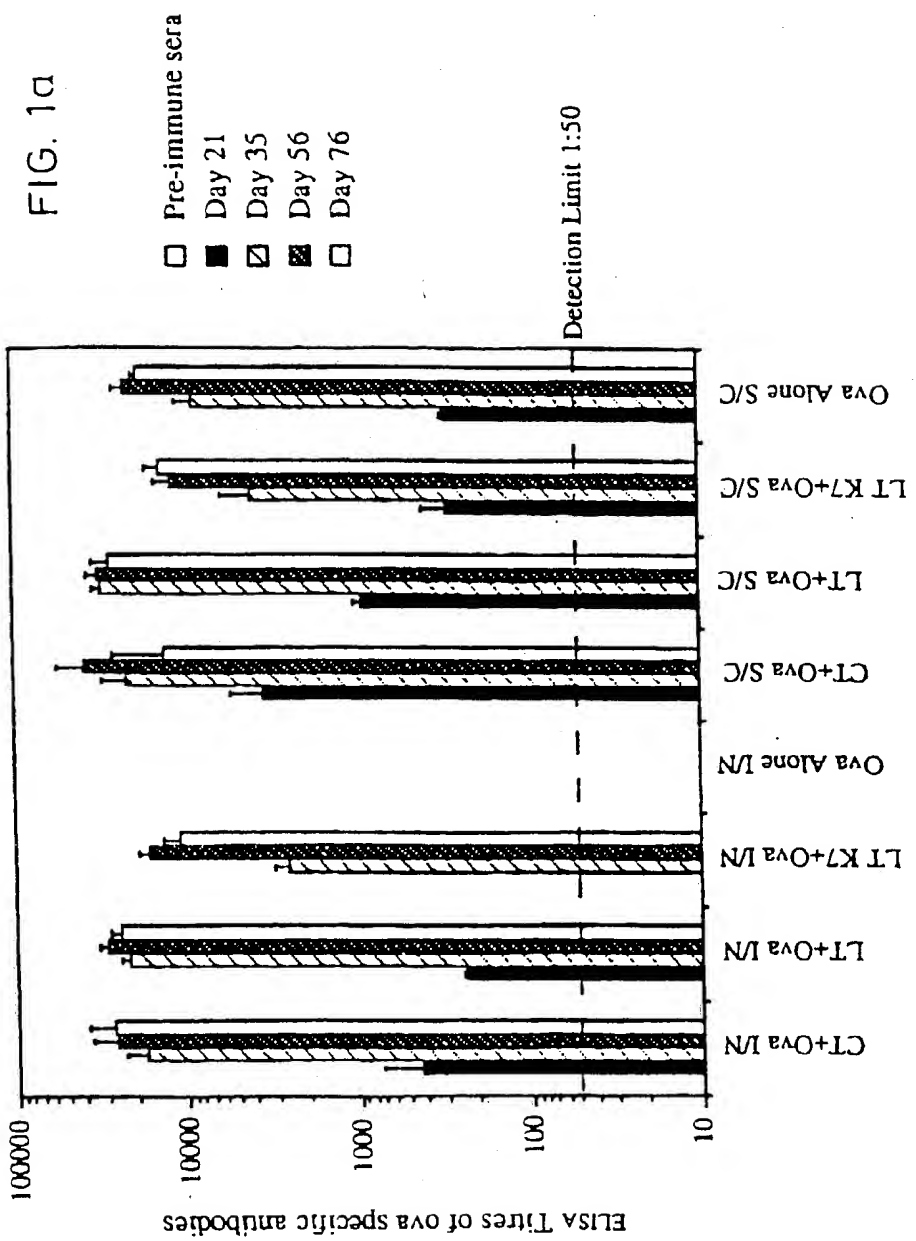
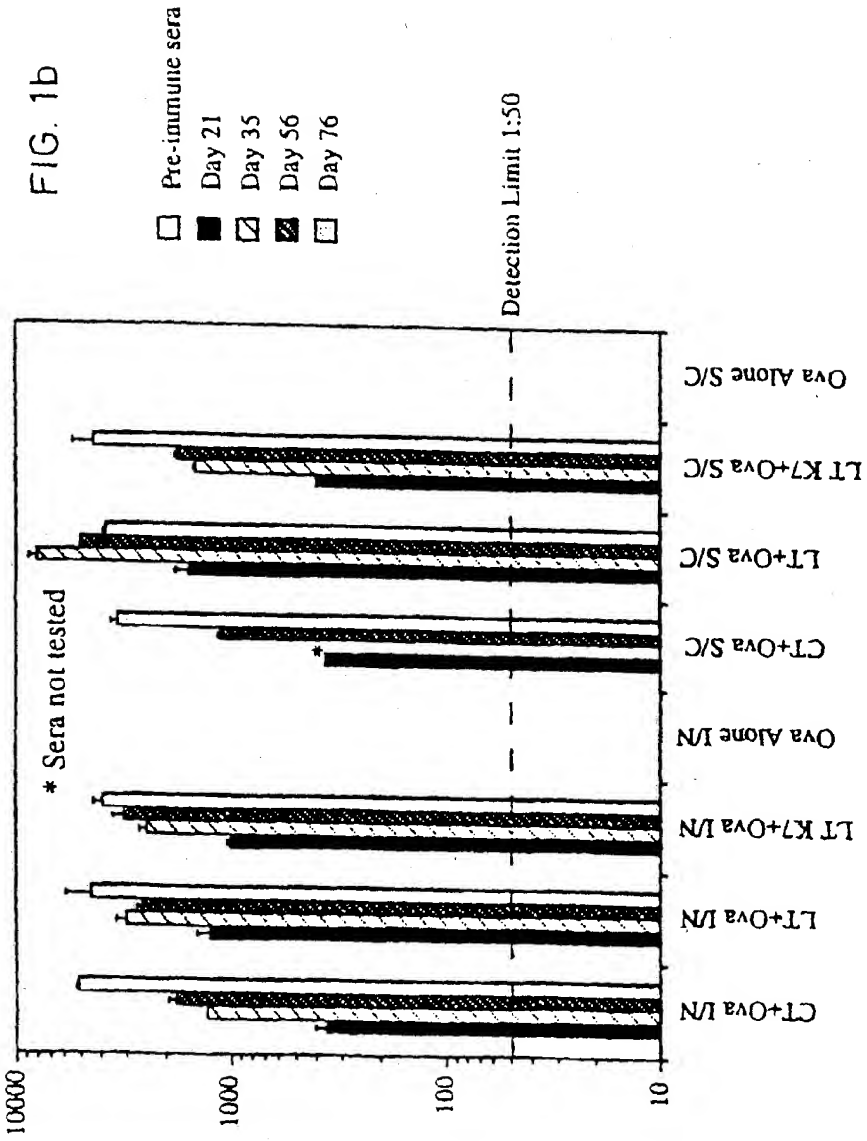

Pre-immune sera
Day 21
Day 35
Day 56
Day 76
Detection Limit 1:50
ELISA Titres of ova specific antibodies
1000000
10000
1000
100
10
CT+Ova I/N
LT+Ova I/N
LT K7+Ova I/N
Ova Alone I/N
CT+Ova S/C
LT+Ova S/C
LT K7+Ova S/C
Ova Alone S/C

FIG. 1b


Pre-immune sera
Day 21
Day 35
Day 56
Day 76
Detection Limit 1:50
* Sera not tested
CT+Ova I/N
LT+Ova I/N
LT K7+Ova I/N
Ova Alone I/N
CT+Ova S/C
LT+Ova S/C
LT K7+Ova S/C
Ova Alone S/C
10000
1000
100
10
ELISA Titre of toxin specific antibodies

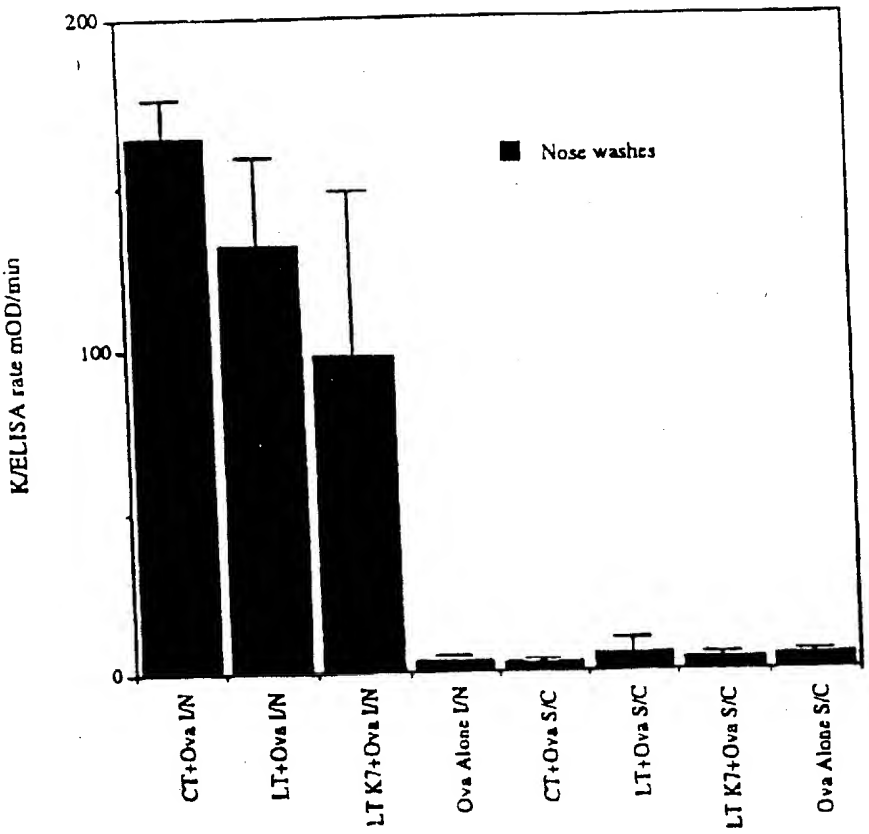

200
100
0
K/ELISA rate mOD/min
Nose washes
CT+Ova I/N
LT+Ova I/N
LT K7+Ova I/N
Ova Alone I/N
CT+Ova S/C
LT+Ova S/C
LT K7+Ova S/C
Ova Alone S/C

FIG. 2(i)

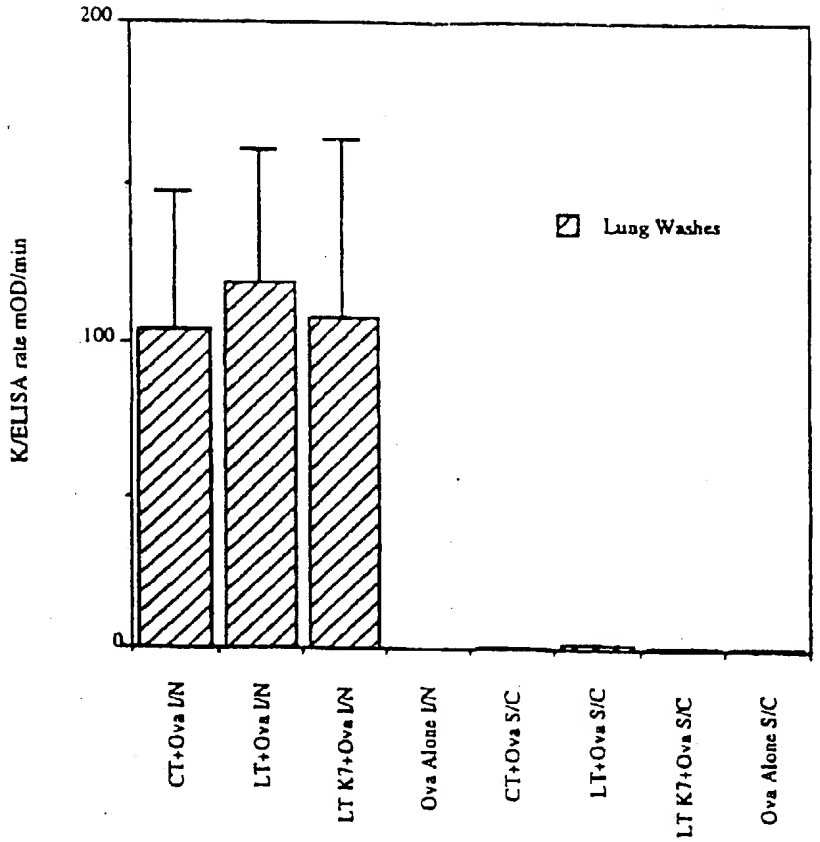

200
100
0
K/ELISA rate mOD/min
Lung Washes
CT+Ova I/N
LT+Ova I/N
LT K7+Ova I/N
Ova Alone I/N
CT+Ova S/C
LT+Ova S/C
LT K7+Ova S/C
Ova Alone S/C

FIG. 2(ii)

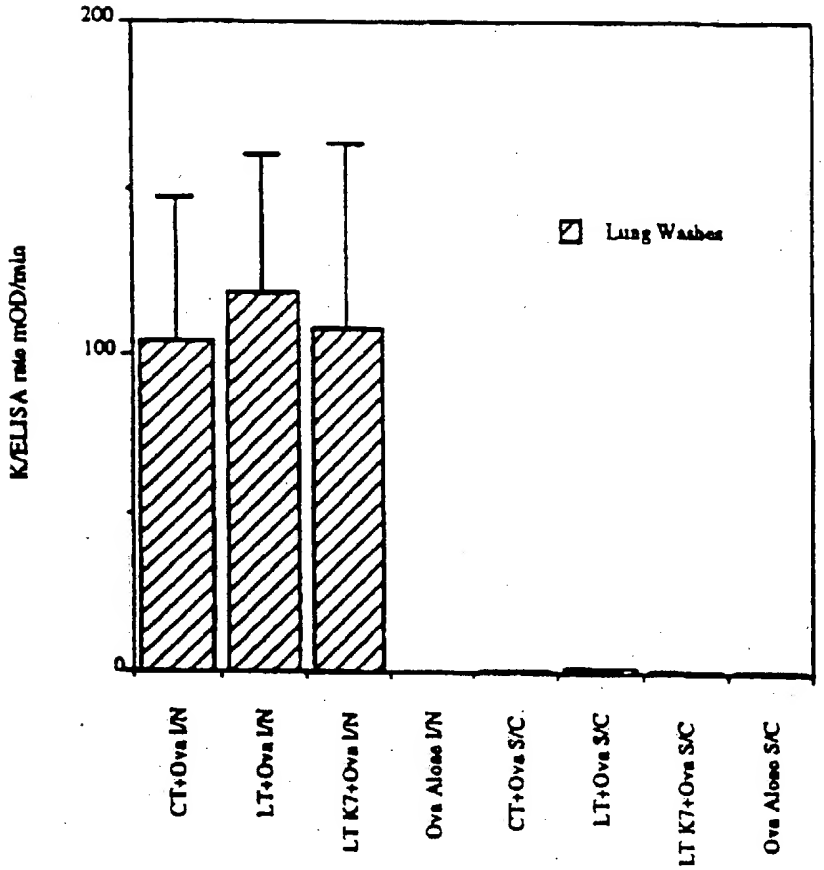


FIG. 2(ii)

FIG. 3

INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/IB 95/00013

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/39

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,92 19265 (AMGEN INC. & UNIVERSITY OF SOUTHERN CALIFORNIA) 12 November 1992 cited in the application see the whole document ---	1-15
Y	VACCINE, vol.11, no.12, September 1993, GUILDFORD, GB pages 1179 - 1184 J. HOLMGREN ET AL. 'Cholera toxin and cholera B subunit as oral-mucosal adjuvant and antigen vector systems.' cited in the application see abstract see page 1182, right column, line 20 - page 1183, right column, line 6 --- -/--	1-15

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 March 1995	04.04.95
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Authorized officer Nooij, F

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